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## RESEARCH ARTICLE

# Hypoxia promotes production of neural crest cells in the embryonic head

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## ABSTRACT

Hypoxia is encountered in either pathological or physiological conditions, the latter of which is seen in amniote embryos prior to the commencement of a functional blood circulation. During the hypoxic stage, a large number of neural crest cells arise from the head neural tube by epithelial-to-mesenchymal transition (EMT). As EMT-like cancer dissemination can be promoted by hypoxia, we investigated whether hypoxia contributes to embryonic EMT. Using chick embryos, we show that the hypoxic cellular response, mediated by hypoxia-inducible factor (HIF)-1 $\alpha$ , is required to produce a sufficient number of neural crest cells. Among the genes that are involved in neural crest cell development, some genes are more sensitive to hypoxia than others, demonstrating that the effect of hypoxia is gene specific. Once blood circulation becomes fully functional, the embryonic head no longer produces neural crest cells *in vivo*, despite the capability to do so in a hypoxia-mimicking condition *in vitro*, suggesting that the oxygen supply helps to stop emigration of neural crest cells in the head. These results highlight the importance of hypoxia in normal embryonic development.

**KEY WORDS:** Hypoxia, HIF-1 $\alpha$ , Chick embryo, Neural crest cells

## INTRODUCTION

The cellular response to hypoxia is a conserved mechanism for cells to tolerate low oxygen availability and adapt to the condition (Semenza, 2013; Taylor, 2008). This adaptive response includes promotion of angiogenesis and glycolysis, and attenuation of cell proliferation (Semenza, 2013). Hypoxia can also induce cancer metastasis by promoting cell dissociation and invasion (Semenza, 2012).

Although hypoxia is generally regarded as a pathological condition, amniote embryos normally develop in a relatively hypoxic environment (Dunwoodie, 2009). In mouse embryos, tissue hypoxia is particularly significant at 8.5 days post coitum (dpc) when the circulatory system is yet to be established (Lee et al., 2001). As amniotes and endothermic organisms, avian embryos are also likely to develop in a hypoxic environment, as the oxygen permeability through the shell and the underlying membranes is extremely low in the first few days of incubation (Kayar et al., 1981). In both mouse and chick embryos, the heart starts beating at the 10-somite stage, which is at 8.5 dpc in mouse and Hamburger and Hamilton (HH) stage 10 in chick (Hamburger and Hamilton, 1951); however, functional blood circulation begins only a day later (Coffin

and Poole, 1988; Kayar et al., 1981; le Noble et al., 2004; McGrath et al., 2003; Meuer and Baumann, 1987; Naňka et al., 2006). At the subcellular level, oxygen consumption is reflected in the morphology of mitochondria. In mammalian and chick embryos of ~10-somite stage, mitochondria show poorly developed cristae of the inner membrane, suggesting that aerobic respiration is not very active (Bancroft and Bellairs, 1975; de Paz et al., 1986; Mackler et al., 1971; Shepard et al., 1998). The cristae become more laminated at later stages, which correlates with the changes from anaerobic to aerobic glycolysis (Mackler et al., 1973; Shepard et al., 1997). Furthermore, the complexity of mitochondrial cristae increases when embryos are grown in higher oxygen (Morris and New, 1979). These observations suggest that early mouse and chick embryos, up to 9 dpc and 2 days old, respectively, are likely to be in a hypoxic state compared with older embryos that have developed aerobic respiration. At these stages, crucial morphogenetic events such as neurulation and initial emigration of neural crest cells in the head take place.

It has been found empirically that culturing mammalian embryos *ex utero* in ambient oxygen concentration causes severe craniofacial malformation (Morris and New, 1979; New, 1978). The oxygen level should be maintained as low as 5% at 6.5–9.5 dpc in mouse embryos, and then increased to 20% or higher only after 9.5 dpc when the neural tube has closed and cranial neural crest cells emigrate out; otherwise normal brain development is compromised and neural crest cells fail to develop (Morris and New, 1979). The stage requiring low (5%) oxygen in mouse embryos corresponds to up to HH stage 14 in chick embryos. In both species, the majority of cranial neural crest cells have emigrated from the neural tube by this stage; only the minority of vagal and trunk neural crest cells continue to emigrate (Baker et al., 1997; Kuo and Erickson, 2011). Hence, the cranial neural crest cells are produced in a limited timeframe when the oxygen availability is relatively low.

In animals, the cellular response to chronic hypoxia is mainly regulated by hypoxia-inducible factors (HIFs) (Semenza, 2001). In addition to angiogenesis and metabolic changes, HIFs upregulate *Snail* (also known as *Snail1*) and indirectly downregulate *E-cadherin* (also known as *Cdh1*), thus promoting epithelial-to-mesenchymal transition (EMT) (Imai et al., 2003; Lester et al., 2007; Luo et al., 2011). HIFs also promote chondrogenesis by inducing *Sox9* (Amarilio et al., 2007). In normoxia, HIF  $\alpha$ -subunits are rapidly degraded as a result of hydroxylation of specific proline residues, which is catalysed by oxygen-dependent prolyl hydroxylases (PHDs). In hypoxia, however, PHDs do not function, hence the  $\alpha$ -subunit of HIF is stabilised and forms a heterodimer with the constitutively expressed HIF  $\beta$ -subunit. The heterodimer binds to specific DNA sequences and upregulates transcription of target genes (Semenza, 2013). Thus hypoxia is reflected by stabilisation of HIF  $\alpha$ -subunits, which themselves can also be

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stabilised by chemical compounds such as dimethylxalylglycine (DMOG) (Elvidge et al., 2006) and a glycine-linked dipeptidyl-quinolone derivative, IOX2 (Chowdhury et al., 2013). Both compounds block oxygen-dependent prolyl-hydroxylation, thus inhibiting degradation of HIF  $\alpha$ -subunits and activating the HIF pathway. DMOG has a broader spectrum of activity, including inhibition of factor-inhibiting HIF (FIH), a second type of HIF- $\alpha$  hydroxylase, the activity of which blocks the interaction of HIF with transcriptional co-activator proteins (Tian et al., 2011).

In early chick embryogenesis, *HIF1A* mRNA, encoding HIF-1 $\alpha$ , is broadly expressed in the head region, whereas *HIF2A* is expressed in extra-embryonic tissues (Ota et al., 2007). One of the downstream target genes of HIF-1 $\alpha$ , phosphoglycerate kinase-1 (*PGK1*), which promotes anaerobic ATP generation (Semenza et al., 1994), is expressed broadly in the head region (Adams et al., 2008), supporting the presence of HIF-1 $\alpha$ -dependent transcriptional activities in the head. Furthermore, targeted deletion of mouse *Hif1a* showed lethality of embryos by 10.5–11.5 dpc, with a shortage of cells in the cranial mesenchyme, a lack of vascularisation and increased cell death in the brain (Iyer et al., 1998; Ryan et al., 1998). These results support the endogenous function and requirement of the HIF-1 $\alpha$ -mediated pathway in normal head development.

The head structures are formed by a large number of neural crest cells. Neural crest cells arise from the interface between the neural plate and surface ectoderm by EMT, then migrate to distant locations and differentiate into a variety of cell types (Le Douarin and Kalcheim, 1999). Induction of the neural crest and subsequent development of neural crest cells are organised by a gene regulatory network, whereby the neural plate border is specified as neural crest and the effector genes function in EMT, emigration, migration and differentiation (Meulemans and Bronner-Fraser, 2004). Key factors for specification of neural crest cells include *Snail1*, *Snail2*, *Sox9*, *Sox10* and *Foxd3*, which are necessary and/or sufficient for emigration of neural crest cells in various species (Cheung et al., 2005; del Barrio and Nieto, 2002; Dottori et al., 2001; Kos et al., 2001; McKeown et al., 2005; Mori-Akiyama et al., 2003; Nieto et al., 1994; Southard-Smith et al., 1998). *Twist1* is also important for EMT in some contexts (Lamouille et al., 2014); however, in mouse embryos, *Twist1* is expressed in migrating neural crest cells as they colonise the branchial arches (Füchtbauer, 1995) whereas it is not expressed in chick neural crest cells (Bothe and Dietrich, 2006).

Among the neural crest specifier genes, *Snail1*, *Sox9*, *Sox10* and *Twist1* are known to be regulated directly by HIF in cancer and/or chondrocytes (Amarilio et al., 2007; Gort et al., 2008; Luo et al., 2011; Steunou et al., 2013; Yang et al., 2008). The chemokine receptor *Cxcr4* is also a HIF-1 $\alpha$ -regulated gene involved in chemoattraction of neural crest cells to their destination (Barriga et al., 2013; Escot et al., 2013; Rezzoug et al., 2011; Staller et al., 2003). A recent study in *Xenopus* showed the role of HIF-1 $\alpha$  for migratory streams of neural crest cells, where Twist induced cell dispersion from the neural plate, whereas *Cxcr4* caused directed migration toward the target (Barriga et al., 2013). Although each of the factors promoted neural crest cell migration individually, co-activation of both factors by stabilised HIF-1 $\alpha$  or hypoxia hindered migration (Barriga et al., 2013), which was due to disruption of neural crest cell clustering that is required in *Xenopus* neural crest cell migration (Nieto, 2011; Theveneau et al., 2010). It was also noted in *Xenopus* embryos that the initial expression of *snail1/2* in the neural crest was not affected by a loss of HIF-1 $\alpha$  (Barriga et al., 2013). Hence, it remains uncertain as to whether HIF-1 $\alpha$  target

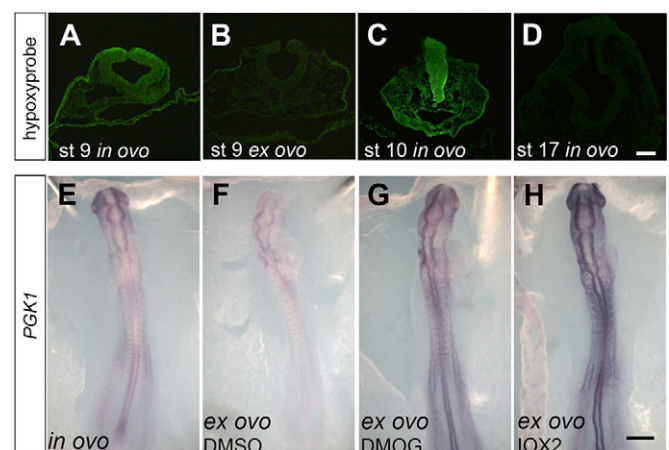
genes identified in pathological contexts are actually employed in embryos or not, and whether hypoxia in the native environment plays an instructive role in normal development.

This study aims to investigate the impact of naturally occurring hypoxia at the pre-circulatory stage in the emigration of head neural crest cells in chick embryogenesis. It should be noted that the formation of trunk neural crest cells is mostly seen after the commencement of vasculogenesis and is not largely affected by high (20–40%) oxygen (Morris and New, 1979) or by deletion of *Hif1a* (Iyer et al., 1998; Ryan et al., 1998). Because of this, the present study focuses on the development of neural crest cells in the head. We demonstrate that emigration of head neural crest cells is attenuated when embryos are exposed to ambient air and is restored by hypoxic cellular response, which is mimicked by loss- and gain-of-function of HIF-1 $\alpha$ , respectively. We also show that the increase of neural crest cells upon hypoxia occurs via promotion of EMT. Lastly, the affected genes are limited to those that are either known targets of HIF-1 $\alpha$  (*SOX9*, *SOX10*, *CXCR4*) or the one implicated in HIF-1 $\alpha$ -mediated EMT (*SNAIL2*), showing the specificity of the effect of hypoxia in neural crest cell development.

## RESULTS

### Early chick embryos *in ovo* are hypoxic and *ex ovo* culture diminishes the hypoxic response

Tissue hypoxia was assessed in chick embryos using pimonidazole, which forms immunologically detectable adducts in hypoxic cells (Arteel et al., 1995; Rademakers et al., 2011; Vukovic et al., 2001). *In ovo* cultured embryos were shown to be positive for pimonidazole at HH stages 9–10 (Fig. 1A,C), similar to mouse embryos at 8.5 dpc (Lee et al., 2001). To test whether incubation of embryos *ex ovo* alters the hypoxic condition, embryos were taken out of the shell at HH stages 5–7, put on agar plates and incubated in ambient air for a further 16 h. Embryos cultured in this way showed much weaker signals, suggesting that they are less hypoxic than embryos cultured *in ovo* (Fig. 1B). It was also noted that embryos that were cultured 1 day longer *in ovo* were not as hypoxic as younger embryos (Fig. 1D). This is in agreement with the commencement of blood



**Fig. 1. Hypoxia in embryos cultured *in ovo* or *ex ovo*.** (A–D) Detection of hypoxia at the midbrain (A,B,D) or hindbrain (C) levels by pimonidazole at indicated stages, cultured *in ovo* (A,C,D) or *ex ovo* for the last 16 h (B). A and B are from the same experiment hence comparable ( $n=6$  for each). Likewise, C and D are comparable ( $n=6$  and 2, respectively). (E–H) Expression of *PGK1* mRNA at stage 12 embryo, cultured *in ovo* (E,  $n=7$ ) or *ex ovo* on agar plates containing DMSO (F,  $n=10$ ), DMOG (G,  $n=5$ ) or IOX2 (H,  $n=5$ ). Scale bars: 100  $\mu$ m (D) and 500  $\mu$ m (H).



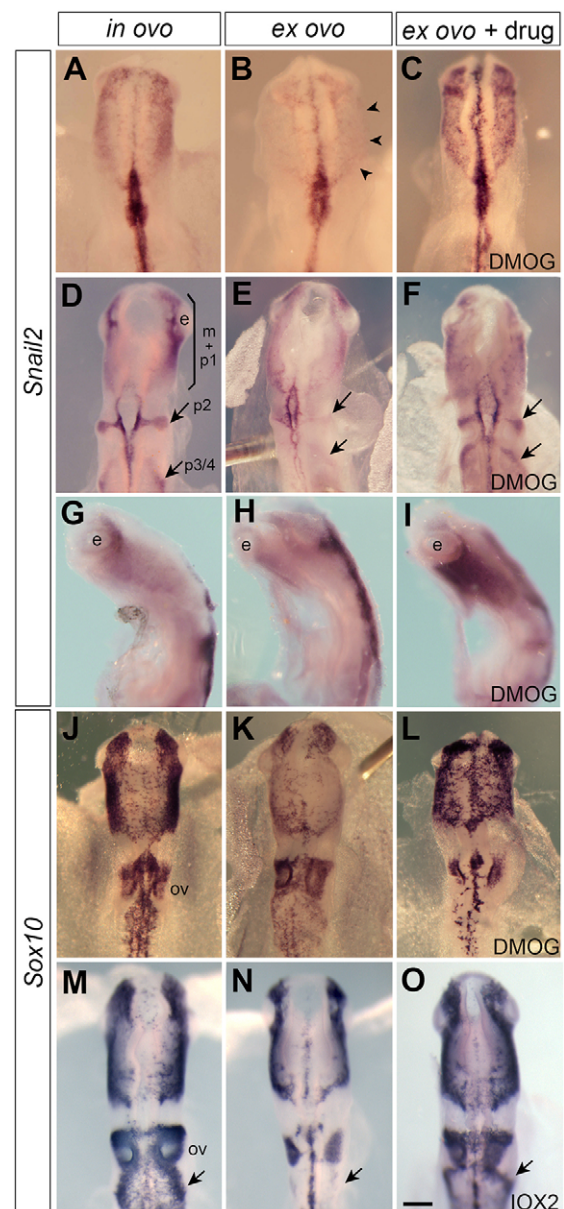
circulation by this stage and subsequent aerobic glycolysis (Mackler et al., 1973; Shepard et al., 1998).

The HIF-1 $\alpha$  target gene *PGK1* was examined in embryos cultured *in ovo* or *ex ovo*. *PGK1* has been identified as a direct target of HIF (Semenza et al., 1994) and indeed its expression is downregulated in *Hif1a*-deleted mouse cells (Higgins et al., 2004, 2007; Iyer et al., 1998; Ryan et al., 1998). *PGK1* was broadly expressed in chick HH stage 12 embryos, although relatively weakly at the trunk level, as described earlier (Adams et al., 2008). Compared with the embryos cultured *in ovo*, embryos cultured *ex ovo* showed weaker expression (Fig. 1E,F). To confirm that the lowered transcription of *PGK1* in *ex ovo* cultured embryos was not due to the culture procedure or malnutrition but rather due to the available oxygen in ambient air, embryos were cultured *ex ovo* with the oxygen-dependent PHD blockers DMOG or IOX2. Compared with the embryos cultured *ex ovo* with vehicle-control DMSO, embryos cultured with DMOG or IOX2 showed stronger *PGK1* expression (Fig. 1G,H). Thus we have three culture conditions: (1) normal incubation *in ovo*; (2) *ex ovo* culture exposed to ambient air with vehicle-control (DMSO) and (3) *ex ovo* with DMOG or IOX2. The *in ovo* condition is regarded as naturally hypoxic, whereas *ex ovo* is presumably hyperoxic compared with the *in ovo* condition and DMOG/IOX2 activates the hypoxic cellular response, thus revealing the effect of hypoxia. Note that control *ex ovo* culture, with or without DMSO, did not result in any difference in the expression of *SOX10* and *PGK1* (Fig. S1).

### Hypoxia is required for emigration of sufficient neural crest cells

Since hypoxia promotes EMT-like cell dissemination in epithelial tumours (Lester et al., 2007; Pennacchietti et al., 2003; Yang et al., 2008), we investigated the production of neural crest cells, the major morphogenetic event caused by EMT, in our different culture conditions. *Ex ovo* cultures were set up at HH stage 5-7 when the neural plate border was being established and neural crest cells were yet to emigrate, and the embryos were incubated for 16-24 h to reach HH stages 10-13. *SNAIL2* expression, which depicts emigrating and migrating neural crest cells (Nieto et al., 1994), was generally weak in *ex ovo* cultured embryos compared with *in ovo* cultured ones (Fig. 2A,B,D,E,G,H). However, the migratory capacity appeared normal because the mesencephalic and metencephalic neural crest cells had migrated ventrolaterally at a similar distance to *in ovo* embryos (Fig. 2A,B). Two streams of myelencephalic neural crest cells, migrating toward 2nd and 3rd/4th pharyngeal arches, where the latter includes cardiac neural crest cells, were not prominent in *ex ovo* cultured embryos (Fig. 2D,E). Notably, DMOG treatment restored *SNAIL2* expression in these neural crest cell populations (Fig. 2C,F,I).

We used another neural crest marker, *SOX10*, which labels neural crest cells for longer periods with partial overlap with *SNAIL2* (Cheng et al., 2000). *SOX10* is also expressed in otic vesicles (Betancur et al., 2011), which can serve as a staining control. Similar to *SNAIL2*, *ex ovo* cultured embryos showed downregulation of *SOX10* in neural crest cells, although the expression in the otic vesicle was not affected (Fig. 2J,K). The migratory capacity of neural crest cells was not affected in the remaining neural crest cells as seen in *SNAIL2*. Embryos treated with DMOG restored *SOX10*-positive neural crest cells, with an increase in late-migrating neural crest cells in the dorsal mesencephalon (Fig. 2L). The same trend was observed with IOX2 (Fig. 2M-O). These results suggest that the cellular response to hypoxia, which naturally occurs *in ovo* or artificially induced by DMOG/IOX2, facilitates emigration of neural crest cells.

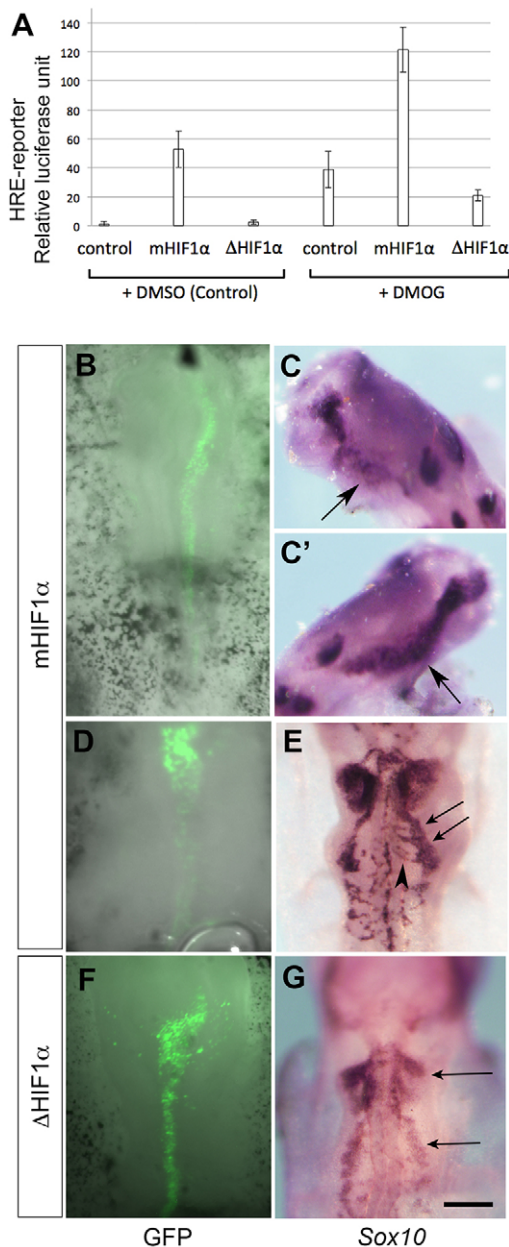


**Fig. 2. Expression of *SNAIL2* and *SOX10* after *ex ovo* culture and DMOG/IOX2 treatment.** (A-I) Expression of *SNAIL2* at 10+ (A-C), 12 (D-F) or 12/13 (G-I) stages in embryos incubated as indicated at the top. Arrowheads in B show the migration-front of neural crest cells that has extended laterally similar to the *in ovo* control. In D, the mesencephalic neural crest cells toward the periocular region and those toward the 1st pharyngeal arch (m+p1) and two distinct streams toward the 2nd (p2) and 3rd/4th (p3/4) pharyngeal arches are indicated. Arrows in D-F indicate p2 and p3/4 streams, showing reduced expression by *ex ovo* culture [E; number of cases presenting the phenotype/all examined cases (n)=23/31] and restoration by DMOG (F, n=17/24). (G-I) Lateral views showing periorbital neural crest cells. (J-O) Expression of *SOX10* at stage 11 (J-L) or 12 (M-O) in embryos incubated as indicated. Neural crest cells from the midbrain are reduced in *ex ovo* cultured embryos (K, n=10/11) compared with the *in ovo* control (J), whereas in DMOG-treated embryos neural crest cells continue to emigrate from the dorsal neural tube (L, n=7/11). Arrows in M-O show the streams toward 3rd and 4th pharyngeal arches that are reduced by *ex ovo* culture (N) and restored by IOX2 (O). e, eye; ov, otic vesicle. Scale bar: 200  $\mu$ m.

### HIF-1 $\alpha$ mediates the increase of neural crest cells

To investigate whether the increased emigration of neural crest cells was mediated by HIF-1 $\alpha$ , chick embryos were introduced with *HIF1A* constructs, which either activated or inhibited the HIF

pathway. The transcriptional activity of the constructs was confirmed using a HIF-responsive-element (HRE) reporter assay (Percy et al., 2006) (Fig. 3A). *HIF1A* containing two mutations



**Fig. 3. The effect of stable and dominant-negative HIF constructs in chick embryos.** (A) *In vitro* reporter assay testing the DNA constructs used in this study. *HIF1A* containing two mutations P402A and P564A (mHIF1α) activates the HIF-responsive element (HRE)-reporter. DMOG activates the reporter by stabilising endogenous HIF-1α, which is repressed by dominant-negative HIF-1α (ΔHIF1α). Results are displayed as relative luciferase activity of triplicates; means±s.d. (B–E) Two embryos electroporated with mHIF-1α on the right side of the neural tube (the electroporated sites are shown in B,D with GFP) and stained for *SOX10*. Arrows in C,C' indicate the group of periocular and maxillary neural crest cells to compare the control (C) and the electroporated (C') sides. Arrows in E show a larger stream of neural crest cells toward pharyngeal arches 3 and 4 compared with the control side. Arrowhead indicates continuous streams of neural crest cells that are not seen on the control side. *n*=24/49. (F,G) An embryo electroporated with ΔHIF-1α on the right side of the neural tube. Arrows in G indicate streams of neural crest cells expressing less *SOX10* than the control side. *n*=4/12. Scale bar: 200 μm.

P402A and P564A, called mHIF-α in this study, is resistant to PHD-catalysed degradation (Hagen et al., 2003) and therefore stably activates the HRE reporter (Fig. 3A). ΔHIF-1α lacks the C-terminal transactivation domain and is unable to bind HIF-1β but is still capable of binding DNA (Jiang et al., 1996; Sutter et al., 2000). Therefore, ΔHIF-1α acts as a dominant negative and was found to attenuate the DMOG-induced HRE reporter activity (Fig. 3A).

Given these results, mHIF-α was overexpressed in the chick embryo on one side of the neural tube by electroporation. *SOX10*-positive cells were increased on the electroporated side in the mesencephalic and metencephalic population (Fig. 3B,C) as well as in the stream toward the 3rd and 4th pharyngeal arches (Fig. 3D,E). In the latter, in addition to the increased amount of neural crest cells in the main stream, a continuous emergence of neural crest cells from the dorsal neural tube was observed even after the majority of cells had emigrated out (Fig. 3E). However, such an increase was not as drastic compared with the electroporation of *SOX9* or *SOX10* (Cheung and Briscoe, 2003; McKeown et al., 2005), which is probably due to the limited availability of HIF-1β subunit *in vivo*. Nevertheless, these results show that the increase of neural crest cells is mediated by stabilised HIF-1α.

To further investigate the role of HIF-1α, endogenous HIF-1α was inhibited by overexpressing ΔHIF-1α. We observed decreased levels of *SOX10* on the electroporated side of the embryo (Fig. 3F,G). Chick embryos were also electroporated with a control GFP construct, which showed symmetrical expression of *SOX10* on the electroporated and control sides of the embryos (data not shown). These results identify the HIF-1α-mediated pathway as the likely molecular mechanism underlying hypoxia-induced emigration of neural crest cells.

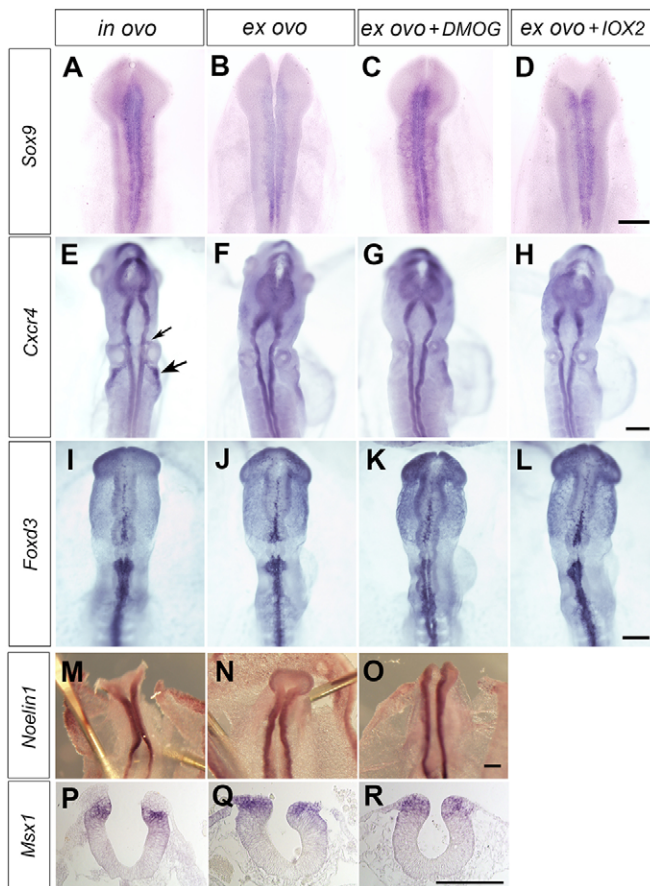
#### The effects of hyperoxia and DMOG/IOX2 are gene specific

The genes that are directly regulated by HIF and expressed in neural crest cells in amniote embryos include *SOX9* (Amarilio et al., 2007), *SOX10* (Steunou et al., 2013), *SNAIL1* (Luo et al., 2011) and *CXCR4* (Staller et al., 2003). Although *SNAIL2* has not been shown as a direct target of HIF so far, a strong correlation of its expression with hypoxia-induced metastatic phenotypes was shown (Wang et al., 2014; Zhang et al., 2013) [note that chick embryo neural crest cells express *SNAIL2*, not *SNAIL1*, whereas in mouse, *Snail1* is the gene expressed in the equivalent structures (Locascio et al., 2002)]. Our *ex ovo* culture showed that, in addition to *SNAIL2* and *SOX10* (Fig. 2), transiently detectable expression of *Sox9* in the emerging neural crest cells was also attenuated by the exposure to ambient air and restored by DMOG or IOX2 (Fig. 4A–D).

To our surprise, expression of another HIF target gene *CXCR4* was abolished almost completely by the *ex ovo* cultures in the streams of neural crest cells toward pharyngeal arches (Fig. 4E,F). This is a far stronger effect compared with *SOX9/10* and *SNAIL2* where we never observed a complete downregulation of the markers. Furthermore, *CXCR4* expression in neural crest cells was not restored by DMOG or IOX2 despite it being a HIF target gene (Fig. 4G,H). A possible explanation is that *CXCR4* requires a high level of HIF-1α proteins that was not achieved by the present method of DMOG/IOX2 application. Other possibilities include a limitation in other factors that are required for *CXCR4* restoration. Given that *CXCR4* expression in the neural tube was not compromised in the *ex ovo* cultured embryos (Fig. 4E–H), the dependence of *CXCR4* expression on HIF is unique to neural crest cells.

By contrast, another neural crest specifier, *FOXD3*, which is regulated by *Pax3* and not by HIF to our knowledge, showed no change after the exposure to ambient air and the subsequent DMOG/





**Fig. 4. Expression of various neural crest markers in embryos cultured *in ovo* or *ex ovo* with compounds.** (A-D) *SOX9* is downregulated by *ex ovo* culture (B,  $n=3$ ) and is restored by DMOG (C,  $n=4$ ) or IOX2 (D,  $n=4$ ). (E) Two streams of *CXCR4*-positive neural crest cells, one toward pharyngeal arch 2 (small arrow) and the other toward pharyngeal arches 3 and 4 (large arrow). These domains of expression are diminished in *ex ovo* cultured embryos (F, completely abolished  $n=4/9$ ; significantly weakened  $n=5/9$ ) whereas the expression in the neural tube is not affected ( $n=8/9$ ). There was no restoration by DMOG (G,  $n=4$ ) or IOX2 (H,  $n=8$ ). I-L show that expression of *Foxd3* is not affected by any culture conditions (J,  $n=5$ ; K,  $n=4$ ; L,  $n=6$ ). Likewise, the expression of *NOELIN1* (M-O; N,  $n=4$ ; O,  $n=7$ ) and *MSX1* (P-R; Q,  $n=3$ ; R,  $n=5$ ) were not affected. *MSX1* expression is shown in sections at the mid/hindbrain level. Scale bars: 200  $\mu\text{m}$ .

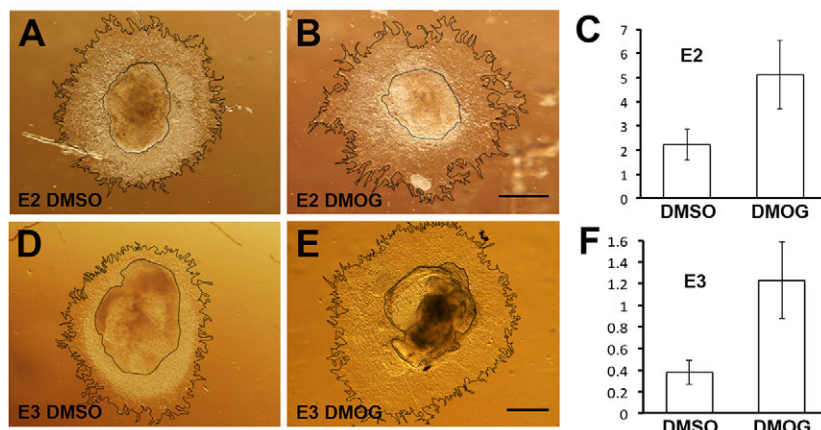
IOX2 treatment (Fig. 4I-L). Likewise, genes upstream of neural crest specifiers, i.e. neural plate border specifiers, *MSX1* (Meulemans and Bronner-Fraser, 2004) and *NOELIN1* (also

known as *OLFMI*) (Barenbaum et al., 2000), were not affected by the *ex ovo* culture and DMOG (Fig. 4M-R). These results show that hypoxia and DMOG/IOX2 are not effective on all genes involved, but rather are limited to specific genes.

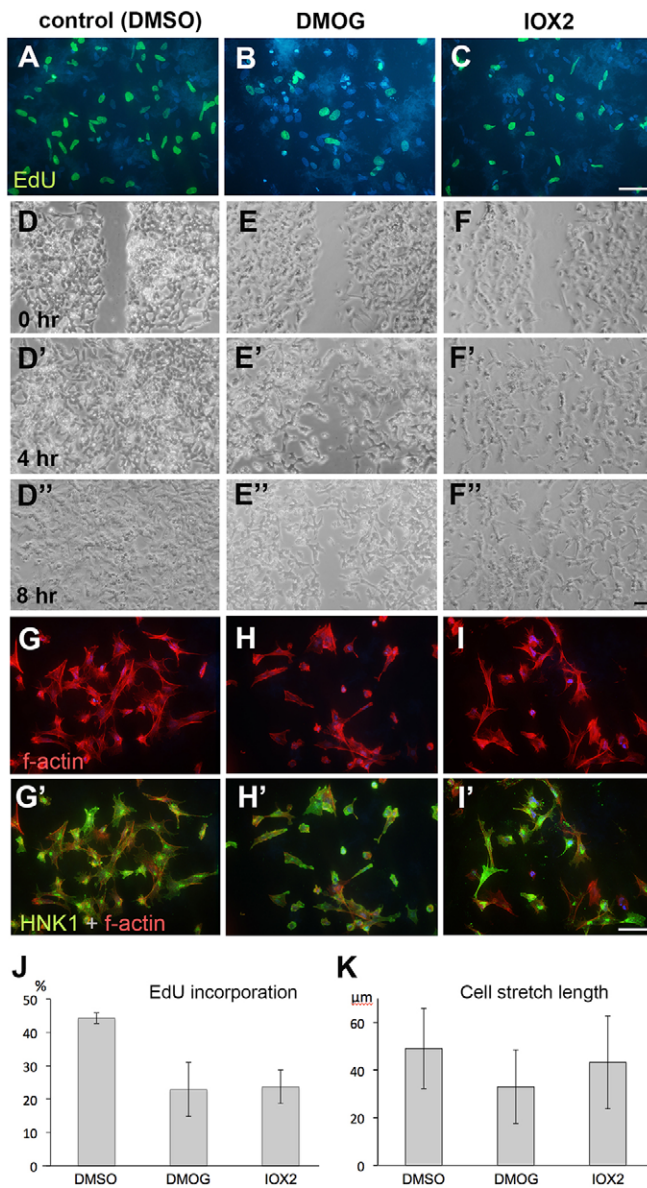
#### Increase of neural crest cells by hypoxia occurs via EMT

The increase of neural crest cells might be caused by an increase of EMT, an increase of cell proliferation and/or a decrease in cell death. The increased *SNAIL2* and *SOX10* expression (Fig. 2) strongly suggests an increase of EMT, as the former triggers EMT by downregulating E-cadherin (Thiery et al., 2009) whereas the latter sufficiently induces EMT-like cell-dissociation of neuroepithelium (McKeown et al., 2005). Hypoxia induces cell cycle arrest (Goda et al., 2003) and apoptosis (Carmeliet et al., 1998) to maintain sustainability; hence, increased cell proliferation and decreased cell death are unlikely. To confirm this, an *in vitro* neural crest cell culture was employed. The neural tube of the midbrain and hindbrain was excised from HH stage 8-9 embryos and cultured for 2 days to allow neural crest cells to emigrate from the explant (Fig. 5A). Some explants were stained with HNK-1 antibody to ensure emigration of neural crest cells (Fig. S2). By culturing with DMOG in the medium, a significantly wider distribution of neural crest cells was observed compared with the control (Fig. 5B,C). This can be caused by increased migratory capacity, increased proliferation and/or increased emergence of neural crest cells from the neural tube.

We next investigated the effect of DMOG and IOX2 on cell proliferation and found that these compounds reduced cell cycle progression, as revealed by lower incorporation of EdU (Fig. 6A-C,J). Therefore, the wider distribution of neural crest cells by DMOG is not due to increased cell proliferation. We also tested the migratory capacity of neural crest cells by scratch assay. DMOG- or IOX2-treated neural crest cells were slow in filling the space compared with the control (Fig. 6D-F). As the cell density affects the gap-filling speed, we further examined the stretch of cells following DMOG or IOX2 treatment. Staining to detect HNK1 and F-actin revealed that cell stretching was mildly affected by DMOG or IOX2 (Fig. 6G-K). Given that neural crest cells of DMOG- or IOX2-treated embryos migrated to the periphery in the same manner as controls *in vivo* (Fig. 2), it is unclear how much the cell morphology impacts the migratory capacity. Hence, the reduced gap-filling speed in the scratch assay is probably due to the slower cell proliferation and/or perhaps reduced cell mobility. These results suggest that the wide spread of neural crest cells caused by DMOG *in vitro* (Fig. 5) is most likely due to the augmented EMT that increased the number of neural crest cells and effectively over-rode the reduced cell proliferation. We suspect that increased neural crest



**Fig. 5. The effect of DMOG on neural crest cells *in vitro*.** (A,B,D,E) Neural tube explants from embryonic day 2 (E2, A, B) at stage 8-10<sup>+</sup> or day 3 (E3, D, E) at stage 14-15<sup>+</sup>, cultured with DMSO (A,D) or DMOG (B,E) for 2 days, showing a spread of neural crest cells. The outline of the neural tube and the spread area of neural crest cells are shown. Scale bars: 500  $\mu\text{m}$ . (C,F) Graphs showing the relative surface area of the spread of neural crest cells compared with the size of neural tube explant (see Materials and methods). Both E2 and E3 explants produce a larger spread of neural crest cells in the presence of DMOG compared with DMSO control ( $P=0.0288$  and  $P=0.0013$ , respectively). The graphs show means  $\pm$  s.d. of  $n=12$  for DMSO and  $n=13$  for DMOG in C and  $n=12$  for both in F.



**Fig. 6. *In vitro* analyses of neural crest cells after DMOG/IOX2 treatment.** (A-C,J) Cell proliferation assay. All cells are stained with Hoechst in blue while cells incorporating EdU are green. (J) Ratio of EdU-incorporating cells (means  $\pm$  s.d.). Cells were counted in at least three independent explants for up to 6000 cells in each of the three groups. Cell proliferation is reduced by DMOG ( $P=0.0032$ ) and IOX2 ( $P=0.0002$ , unpaired Student's *t*-test). (D-F) Cell scratch assay. Three time points (0, 4 and 8 h after scratching) are shown for each group of cells. Control neural crest cells show a rapid gap-filling within 4 h ( $n=6$ ) whereas DMOG- or IOX2-treated cells are relatively slow ( $n=7$  for each). (G-I,K) Cell morphology assay. Cells are stained for F-actin (red; G-I) and HNK-1 (green; G'-I'). The longest axes of individual HNK-1-positive cells were measured in 200 cells from two independently obtained samples and the quantification is shown in K (means  $\pm$  s.d.). Cell extension is affected by DMOG ( $P<0.0001$ ) and IOX2 ( $P=0.0021$ , unpaired Student's *t*-test). Scale bars: 50  $\mu$ m.

cells emerged at the expense of the neural tube, as seen in *SOX10*-overexpressing embryos (McKeown et al., 2005) and/or by prolonged emigration of neural crest cells.

### Hypoxia has the potential to prolong production of neural crest cells

Although the onset of neural crest cell migration has been studied in detail (Meulemans and Bronner-Fraser, 2004), little is known about

the mechanism whereby the neural crest cells stop emerging. Most pre-otic neural crest cells stop emigrating from the neural tube by HH stage 12 (Baker et al., 1997; Lumsden et al., 1991), whereas the vagal neural crest cells, including cardiac neural crest cells, continue to emigrate at the transition between the cranial and trunk parts (Kuo and Erickson, 2011; Reedy et al., 1998). At HH stage 13, the endothelial network begins to form in the head region (Coffin and Poole, 1988), which is followed by the establishment of functional blood circulation via vitelline arteries and veins at stage 16/17 (le Noble et al., 2004). Given the narrow timeframe of neural crest cell emigration, which coincides with the absence of functional blood circulation, we hypothesised that commencement of blood circulation might contribute to the cessation of neural crest cell emigration. To test this, we challenged the ability of the old-stage neural tube to produce neural crest cells by activating the hypoxic response. Thus, we examined whether the discontinuation of neural crest cell emigration is intrinsically programmed or if it is regulated by the oxygen supply.

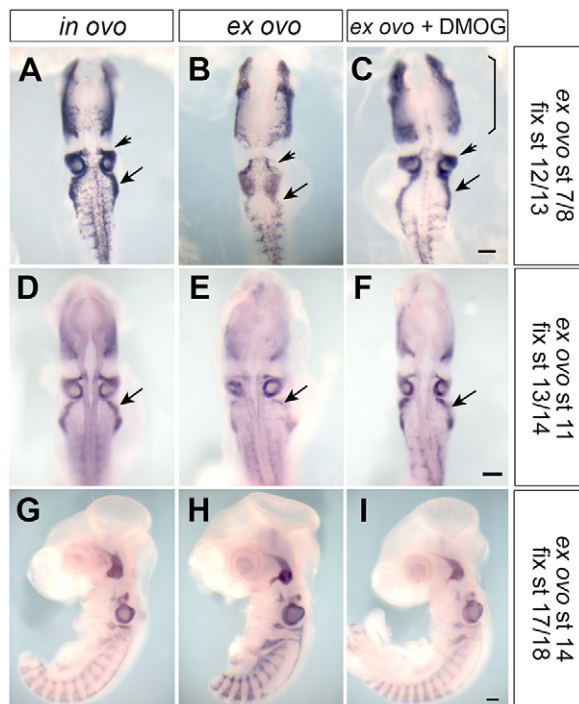
We first performed explant assays using hindbrain from 3-day-old embryos (HH stage 14-15<sup>-</sup>). A substantial amount of neural crest cells emigrated out of the neural tube explant (Fig. 5D), although not as many as in the day 2 embryos. Interestingly, DMOG increased the amount of neural crest cells from the old-stage explants as in young neural tube explants (Fig. 5E,F). Thus, the neural tube at embryonic day 3 is still capable of producing neural crest cells *in vitro*, which is augmented by the cellular response to hypoxia. This result suggests that the timeframe of hypoxia *in vivo* forms the temporal limit for neural crest cells to undergo EMT.

Next, to examine whether hypoxia can prolong neural crest cell emigration *in vivo*, embryos were treated with DMOG at relatively late stages (Fig. 7). At HH stage 7-8, the neural crest border is specified but neural crest cells are yet to emigrate out (Basch et al., 2006). At HH stage 11 in the midbrain, early-migrating neural crest cells have already left the neural tube and the late-migrating ones are just finishing emigration (Baker et al., 1997), whereas in the hindbrain neural crest cells are actively emigrating (Lumsden et al., 1991). At HH stage 14, cranial neural crest cells have migrated out, although the neural tube is still able to produce neural crest cells *in vitro* and is able to respond to DMOG as seen in Fig. 5D-F. Embryos at each of these stages were cultured *ex ovo* with either DMSO or DMOG for a further 24 h.

The results of *ex ovo* cultures at HH stages 7-8 were similar to those of stages 5-7, as shown in Fig. 2: downregulation of *SOX10* by the *ex ovo* cultures and restoration by addition of DMOG (Fig. 7A-C). Embryos excised at stages 11 and cultured *ex ovo* presented clear effects in the neural crest streams toward the pharyngeal arches, whereas mesencephalic and metencephalic neural crest cells were only mildly affected (Fig. 7D,E). The phenotype was restored by the presence of DMOG (Fig. 7F). However, embryos cultured *ex ovo* at stage 14 did not show any difference from *in ovo* cultured embryos and no additional neural crest cells were produced by culture with DMOG (Fig. 7G-I) despite its ability to do so *in vitro* (Fig. 5D-F). Thus, DMOG-induced stabilisation of HIF-1 $\alpha$  was not sufficient to produce *de novo* neural crest cells *in vivo* at such late stages.

To further examine the prolonged emigration of neural crest cells and the competence of the neural tube to respond to DMOG, emerging neural crest cells were labelled at various stages by Dil injection into the neural tube, such that only newly emigrated neural crest cells would be labelled following the injection (Fig. 8). Dil injection at stage 10 *in ovo* and following 14 h of incubation labelled three migratory streams lateral to rhombomeres 2, 4 and 6, as well as a relatively small amount of neural crest cells from the midbrain

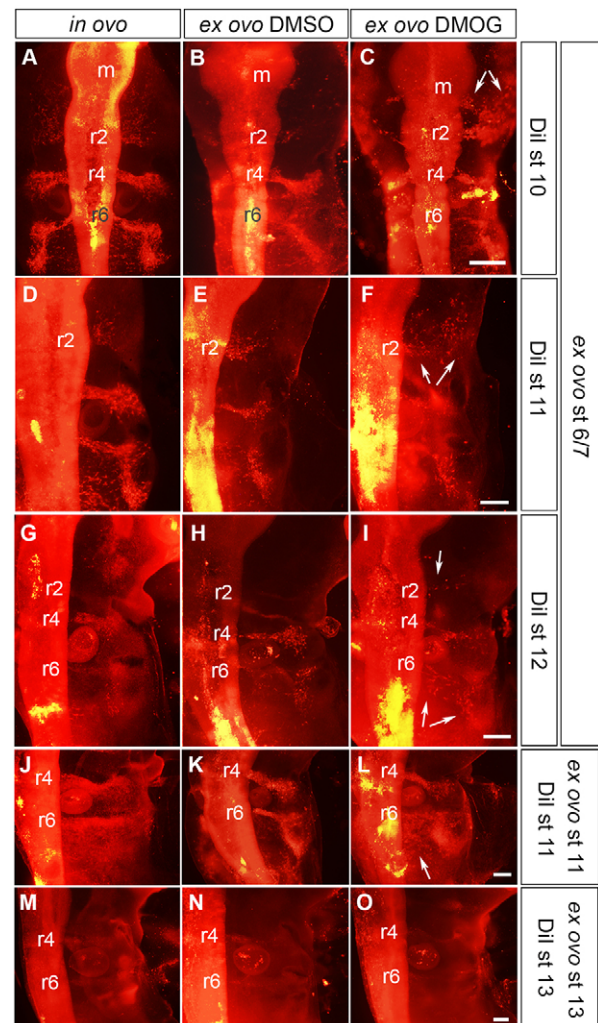




**Fig. 7. The plasticity and commitment in production of neural crest cells in response to DMOG.** All *ex ovo* embryos were incubated overnight to reach stages 12/13, 13/14 and 17/18, respectively. (A-C) Embryos cultured *ex ovo* from stage 7/8 (B,  $n=3/4$ ) show downregulation of SOX10 in two streams of neural crest cells; toward pharyngeal arches 2 (small arrows) and 3 and 4 (large arrows), compared with *in ovo* cultured embryos (A), which are restored (C;  $n=3/4$ ). There is also increased expression in neural crest cells migrating toward the pericardial, maxillary and mandibular regions (bracket in C) compared with B. The results are similar to those shown in Fig. 2 in which *ex ovo* culture was started at stages 5-7. (D-F) Downregulation of SOX10 by *ex ovo* culture at stage 11 (E,  $n=9/12$ ) is limited to the neural crest stream toward the pharyngeal arches 3 and 4 (arrows), which is restored by DMOG (F,  $n=9/12$ ). Other neural crest streams do not show obvious changes. (G-I) Embryos cultured *ex ovo* from stage 14 do not show significant downregulation of SOX10 (H,  $n=13/13$ ) and are not affected by DMOG (I,  $n=16/16$ ). Scale bars: 200  $\mu\text{m}$ .

(Fig. 8A). Embryos cultured *ex ovo* from stage 6/7 and injected with DiI at stage 10 showed a reduction in labelled neural crest cells from the mesencephalon and rhombomere 2 (Fig. 8B). DMOG restored neural crest cells from those sites, where both newly emerging and already distantly migrated ones were labelled (Fig. 8C). A similar restoration was observed by injecting DiI at stage 11 at the level of rhombomere 2 (Fig. 8D-F). Hence, the additional neural crest cells seen in Fig. 2 and Fig. 3B-E are likely to be due to the continuous emigration of neural crest cells with full migratory capacity rather than delayed migration of early despatched cells. DiI injection into *in ovo* embryos at stage 12 followed by incubation for 14 h only showed migrated neural crest cells at the level of rhombomere 2 and no further emigration from this level (Fig. 8G). This was also the case for *ex ovo* cultured control embryos (Fig. 8H). However, DMOG-treated embryos showed migrating neural crest cells lateral to rhombomere 2, revealing new emigration of neural crest cells from the neural tube after stage 12 (Fig. 8F). Hence, DMOG-treated embryos are able to produce neural crest cells newly from rhombomere 2 at stage 12 when control embryos have ceased the production.

We have further challenged the ability of the neural tube to produce neural crest cells in response to DMOG at later stages.



**Fig. 8. DiI labelling of the neural tube examining continuous emigration of neural crest cells and the competence to respond to DMOG at various stages.** (A-I) Examining continuous emigration of neural crest cells. Embryos were either cultured *in ovo* (A,D,G) or set for *ex ovo* cultures at stages 6-7 and then injected with DiI at stages 10 (A-C, 10 somites, except B, which was at 9 somites), 11 (D-F, 12 somites) or 12 (G-I, 17 somites) followed by further overnight incubation. DiI injection at stage 10 or 11 has labelled neural crest cells from the posterior midbrain (m) and rhombomere (r) 2 *in ovo* (A,  $n=11$ ; D,  $n=10$ ), as well as in *ex ovo*, more significantly in DMOG-treated embryos (C,  $n=8$ ; F,  $n=8$ ) compared with DMSO control (B,  $n=7$ ; E,  $n=8$ ). Arrows in C and F indicate neural crest cells of both newly emigrated and further migrated ones away from the neural tube. Following DiI injection at stage 12, emigrating cells were not detected from r2 *in ovo* (G) and *ex ovo* control (H), whereas treatment with DMOG showed DiI-labelled neural crest cells from r2 as well as increased emigration from r6 (I, arrows). (J-O) Competence of embryos to respond to DMOG for neural crest cell emigration. Embryos were cultured either *in ovo* (J,  $n=11$ ; M,  $n=3$ ) or *ex ovo* with control DMSO (K,  $n=4$ ; N,  $n=5$ ) or DMOG (L,  $n=5$ ; O,  $n=5$ ) at stages 11 (12 somites) or 13 (18 somites) and injected with DiI, followed by further overnight incubation. Among embryos injected at stage 11, *ex ovo* cultured control (K) show reduced neural crest cells compared with *in ovo* cultured ones (J), whereas embryos exposed to DMOG have restored the emigration of neural crest cells from r6 and posterior (L, arrow). Following injection at stage 13, few neural crest cells have newly emerged in *in ovo* (M), DMSO-treated (N) or DMOG-treated (O) embryos. Scale bars: 200  $\mu\text{m}$ .

*Ex ovo* cultures followed by immediate DiI injection at stage 11 resulted in reduction of neural crest cells compared with *in ovo* cultures (Fig. 8J,K), which was restored by DMOG, especially at the posterior hindbrain (Fig. 8L), consistent with the result seen in Fig. 7F. However, at stage 13 when *in ovo* cultured embryos showed



little labelling of newly emerged neural crest cells, DMOG-treated embryos did not show new emergence of neural crest cells either (Fig. 8M–O). Together with the result of Fig. 7, it became apparent that the *de novo* emigration of neural crest cells caused by DMOG is limited to only a short while longer than the normal emigration period, such as that seen at stage 12 in rhombomere 2. This is probably due to the advanced differentiation commitment of neural tube cells and/or the overwhelming oxygen supply by blood circulation *in vivo*.

All together, our results demonstrate that stabilization of HIF-1 $\alpha$  promotes expression of neural crest specifier genes *SNAIL2*, *SOX9/10* and *CXCR4*, which augment EMT thus leading to the sufficient emigration of neural crest cells.

## DISCUSSION

### Hypoxia in normal embryogenesis: species specificity

One difference between aquatic vertebrates and amniotes is their responsiveness to low oxygen, with the former being more tolerant. Zebrafish embryos at gastrula and neurula stages generally develop normally in anoxia (0% oxygen) for 24 h, as well as in 20% oxygen (Padilla and Roth, 2001). In addition, aquatic animals in the natural environment withstand wide swings in water temperature that affect oxygen consumption, as well as the solubility of oxygen in the water. By contrast, amniote embryos grow at a stable temperature and are vulnerable to abnormally low or high temperatures. This suggests that the role of the HIF pathway in development might be different between amniotes and aquatic vertebrates. In amniotes, the sustained low level of oxygen activates the HIF pathway, thus contributing to normal developmental events such as vasculogenesis (Naňka et al., 2006), whereas aquatic vertebrate embryos utilise the HIF pathway for the adaptive response to low oxygen. In fact, in zebrafish embryos, the HIF pathway is crucial for hypoxic preconditioning, where mild hypoxia protects the body against more severe hypoxia that may occur later (Manchenkov et al., 2015).

Recent studies support a role for the HIF pathway in developing neural crest cells in *Xenopus* and zebrafish embryos (Barriga et al., 2013); however, the requirement and function might differ between species. In addition to the difference in the developmental conditions as mentioned above, the mechanism of neural crest development also differs between amniotes and aquatic vertebrates. An example is the role of *Twist1* and *Snail1/2* and the effect of hypoxia on *Snail2*. *Twist1* is a known HIF target gene in some contexts (Gort et al., 2008; Yang et al., 2008) and, in neural crest cells, it plays more crucial roles in aquatic embryos than in amniotes. In *Xenopus* embryos, *Twist* functions to disperse neural crest cells thus facilitating EMT (Barriga et al., 2013). However, in mouse embryos, deletion of *Twist1* in neural crest cells does not affect their induction or migration (Bildsoe et al., 2009) while, in chick, *TWIST1* is not expressed in neural crest cells (Bothe et al., 2007). With regard to *snail2*, a loss of HIF-1 $\alpha$  in *Xenopus* affects migration of neural crest cells without affecting the expression of *snail2* (Barriga et al., 2013). Whereas in the chick, *SNAIL2* expression is broadly downregulated by the exposure to ambient air (Fig. 2) reflecting the reduced EMT, which is in agreement with the observation that *SNAIL2* is necessary and sufficient for the specification of cranial neural crest cells and their subsequent EMT (del Barrio and Nieto, 2002; Nieto et al., 1994). Another species difference is that neural crest cells in *Xenopus* embryos require clustering for ‘collective migration’ whereas chick embryos do not (Nieto, 2011). In experimental conditions where HIF-1 $\alpha$  or *Twist* is downregulated, *Xenopus* neural crest cells maintain

E-cadherin expression and yet migrate to the periphery (Barriga et al., 2013), whereas in chick embryos E-cadherin-positive neural crest cells form aggregates and do not migrate (Rogers et al., 2013). Differences in the mechanism for neural crest cell induction and migration between species reflect different roles each gene may have and perhaps the role of hypoxia as well.

### The role of HIF-dependent pathway in the gene regulatory network of neural crest cells

In this study, genes affected by ambient air were *SNAIL2*, *SOX10*, *SOX9* and *CXCR4*. Whereas both *Sox9* and *Sox10* have been identified as HIF target genes (Amarilio et al., 2007; Steunou et al., 2013; Zhang et al., 2011) and are sufficient to produce neural crest cells at least in the trunk neural tube (Cheung and Briscoe, 2003; McKeown et al., 2005), these genes have different expression profiles and functions. *Sox9* is transiently expressed in the dorsal neural tube at the neural crest-emigration stages but not required for the emigration *per se* (Mori-Akiyama et al., 2003). Later on, *Sox9* functions to specify the chondrogenic lineage. By contrast, *Sox10* is expressed in migrating neural crest cells, is required for induction and migration of neural crest cells (Southard-Smith et al., 1998) and later functions to promote glial differentiation (Britsch et al., 2001). Together with *Cxcr4*, the receptor for the chemo-attractant SDF1, all known HIF target genes expressed in neural crest cells in addition to *Snail2* are shown to be vulnerable to ambient air in this study.

Among the genes of the neural crest regulatory network (Meulemans and Bronner-Fraser, 2004), *FOXD3*, *NOELIN1* and *MSX1* were not responsive to the exposure to ambient air or DMOG/IOX2 treatment (Fig. 4), demonstrating the specificity of the effect of hypoxia in the gene regulatory network. In agreement with our result, neural tube explants from *Foxd3* mutant mice produce fewer neural crest cells than control explants *in vitro*; nonetheless, under hypoxia, such *Foxd3* mutant neural tubes are able to produce a large amount of neural crest cells as in the wild-type neural tube (Pfaltzgraff et al., 2012). Thus, the competence of neural crest cell precursors to respond to hypoxia and undergo EMT is not compromised by the lack of *Foxd3*. *Foxd3* is sufficient to induce emigration of neural crest cells in chick and mouse (Dottori et al., 2001; Kos et al., 2001; Teng et al., 2008). Hence, when embryos are exposed to ambient air, the HIF-dependent pathway is attenuated but the neural tube can still produce a certain amount of neural crest cells thanks to the expression of HIF-independent genes. It appears that a combination of HIF-dependent and HIF-independent pathways provides sustainability in producing neural crest cells even in conditions with variable oxygen availability. This might be particularly important in aqueous vertebrate embryos that bear changeable oxygen availability, and in the spinal cord of amniotes where neural crest cells emigrate when the blood circulation has started. In fact, mouse embryos lacking *Foxd3* in the neural crest exhibit severe defects at the trunk level with a complete loss of peripheral nerves, in contrast to the head region where only a mild reduction of neural crest cells is observed (Teng et al., 2008). These data suggest that production of neural crest cells is controlled by a balanced mix of genes, hypoxia-sensitive and insensitive ones, depending on the axial level and species.

### Hypoxia and temporal regulation for ceasing neural crest emigration

The head neural crest cells mostly emigrate out of the neural tube by HH stage 12 in chick embryos (Baker et al., 1997; Lumsden et al., 1991). In spite of this, our result using explant culture demonstrated that the head neural tube from older embryos is capable of

producing neural crest cells *in vitro*, which is enhanced by DMOG treatment (Fig. 5). This result is in agreement with other studies that observed increased dissociation of neural crest cells from day 12 rat embryos (equivalent to chick embryonic day 3 and 9–10 dpc in mouse) that were cultured at low oxygen concentration (Studer et al., 2000). These findings, among other *in vitro* studies (Morrison et al., 2000) support the idea that hypoxia promotes production of neural crest cells. *In vivo*, DMOG treatment at early stages induced continuous emigration of neural crest cells (Figs 2, 7 and 8), whereas at later stages, DMOG only restored the neural crest cells that had been reduced by ambient air, thus, not over-riding the endogenous programme of neural crest cell production (Fig. 7 and Fig. 8J–O). The inability of the old neural tube to produce additional neural crest cells at late stages in the head might be partly because of the progressive neural tube development and commitment in differentiation. In addition to this, we propose that it might also be due to the high oxygen demand for oxidative metabolism in response to rapid growth and increasing energy consumption (Mackler et al., 1971, 1973; Shepard et al., 1998, 2000), which may not allow the HIF pathway to function. This is in contrast to young embryos that successfully produce a large number of neural crest cells in the head region thanks to the hypoxic conditions. Indeed, HIF-1 $\alpha$  is required for producing neural crest cells in young embryos as shown in Fig. 3. It is tempting to suggest that the increasing availability of oxygen in the tissues may contribute, at least in part, to the cessation of neural crest cell production in the head.

## MATERIALS AND METHODS

### Ex ovo culture

The experimental procedure for handling chick embryos was locally approved (UB/14/050). The *ex ovo* culture of chick embryos was performed on agar plates as described (Chapman et al., 2001). Agar plates were made with 0.4% DMSO (Sigma), 2 mM DMOG (Cayman Chemicals) with 0.1% DMSO or 0.4 mM IOX2 (Sigma) with 0.4% DMSO. Throughout this study the embryos were staged according to the number of somites following the Hamburger and Hamilton (1951) staging method and comparisons were made on embryos with same somite numbers or one-somite different embryos.

### Hypoxyprobe assay

Pimonidazole HCl (Hypoxyprobe) was dissolved to 300  $\mu$ M in Hanks' solution and 20  $\mu$ l injected into stage 9/10 or stage 17 embryos *in ovo*, underneath the vitelline membrane. Embryos were incubated for 30 min before fixation with 4% paraformaldehyde (PFA) in PBS and processed for cryosectioning. The sections were immunostained with FITC-conjugated anti-pimonidazole antibody (Hypoxyprobe, clone 4.3.11.3, 1:50).

### RNA *in situ* hybridisation

RNA *in situ* hybridisation was performed as described previously (Amirthalingam et al., 2009). The same batches of embryos cultured in different conditions (Fig. 1E–H, for example) were processed in parallel under the same conditions to make them comparable between groups. The origins of DNA templates for RNA probes were: *PGK1* (ARK genomics, EST91d8), *SNAIL2* (A. Nieto, Instituto de Neurociencias, Spain), *SOX10* (P. Scotting, University of Nottingham, UK), *SOX9* (M. Cheung, University of Hong Kong), *CXCR4* (ARK genomics, EST877b1), *FOXD3* and *NOELINI* (M. Bronner, Caltech, USA), *MSX1* (T. Nohno, Kawasaki Medical School, Japan).

### Reporter assay and electroporation

Transcriptional activity of DNA constructs (mHIF-1 $\alpha$  and  $\Delta$ HIF-1 $\alpha$ ) was assessed using the Dual-Luciferase Reporter Assay System (Promega) using HEK-293 (ATCC). mHIF-1 $\alpha$  was a gift from C. Taylor (University College Dublin, Ireland).  $\Delta$ HIF-1 $\alpha$  was made by deleting the C-terminal half at the

A/III site (Jiang et al., 1996). HIF-1 $\alpha$  construct DNA or empty vector plasmid (0.5  $\mu$ g/well) were co-transfected with a HRE reporter (gift from F. S. Lee, University of Pennsylvania, USA) (Wenger et al., 2005) at 0.45  $\mu$ g/well, along with TK *Renilla* luciferase at 0.02  $\mu$ g/well (Promega) in 24-well plates in triplicate. At 24 h post transfection, cells were treated with either 0.05% DMSO or 1 mM DMOG and incubated for 24 h before lysing the cells for the luciferase assay. *In ovo* electroporation was performed as described previously (Itasaki et al., 1999).

### In vitro neural crest cell culture

Neural tube explants were excised from the midbrain or hindbrain at stage 8–10<sup>−</sup> or hindbrain at stage 14–15<sup>+</sup> and treated with dispase as described previously (Amirthalingam et al., 2009). To anchor each explant to the culture dish, 20  $\mu$ l of collagen type-I (BD Biosciences) was used. Once the collagen was set, 500  $\mu$ l DMEM/F-12 containing either 1 mM DMOG or 0.05% DMSO was added to each well. Explants were cultured at 37°C and 5% CO<sub>2</sub> for 48 h, after which explants were fixed with 4% PFA for 15 min and the images were taken using a stereo-microscope. On each image, the surface area (*S*) of the spread of neural crest cells including the explanted neural tube and the surface area of the neural tube were measured by ImageJ software. For each case, the ratio of [*S*(spread of neural crest cells including neural tube explant)−*S*(neural tube explant)]/*S*(neural tube explant) was calculated and averaged within each group. An unpaired *t*-test was calculated using GraphPad Prism 6 software.

### Cell proliferation, morphology and scratch assays

Neural tube explants were cultured as above on fibronectin-coated coverglass. After 48 h, the covering collagen and neural tube were removed and the medium was changed to that containing DMSO 0.2%, 1 mM DMOG or 0.2 mM IOX2 and incubated for a further 24 h. For the cell proliferation assay, cells were incubated with EdU (Molecular Probes, C10637) for 6 h following the manufacturer's protocol. For the scratch assay, scratches were made using pipette tips and photos were taken immediately after scratching and following 4 and 8 h. For cell elongation assay, cells were fixed and stained with phalloidin (Invitrogen, A12380, 1:2000) and HNK-1 (Invitrogen, 180167Z, 1:80) which was visualised with Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, A10667, 1:1000). The longest stretch length was measured using Photoshop software (Adobe).

### DiI injection

DiI18(3) (DiI) solution was prepared by mixing one part of 0.5% DiI in ethanol and 9 parts of 10% sucrose in H<sub>2</sub>O and was injected into the neural tube at relevant stages. Injected embryos were incubated for 14–16 h before observation. For *in ovo* controls, 3 ml albumin was taken and a window was opened on the shell for access to the embryos. The window was sealed with tape for further incubation.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

N.I. and D.F.H. designed the experiments; D.S., E.K., E.B. and P.K. performed the experiments and analysed the data; N.I. drafted the manuscript. D.S., D.F.H. and N.I. edited the manuscript.

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### Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.131912/-/DC1>



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